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I further certify that the above application is now proceeding in the name of THE UNIVERSITY OF NEWCASTLE RESEARCH ASSOCIATES LIMITED pursuant to the provisions of Section 104 of the Patents Act 1990.

WITNESS my hand this
Seventeenth day of August 2005

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**Patents Act 1990**


**PROVISIONAL SPECIFICATION**

for the invention entitled:

"A Method of Treatment and Agents Useful for Same"

The invention is described in the following statement:

# A METHOD OF TREATMENT AND AGENTS USEFUL FOR SAME

The present invention relates generally to a method of modulating the growth of cells and, more particularly, to a method of inhibiting the growth of neoplastic cells. Still more particularly, the present invention is directed to a method of down-regulating the growth of neoplastic epithelial cells. The present invention is useful, *inter alia*, in the therapeutic and prophylactic treatment of cancers such as, but not limited to cancers of the breast, colon, skin, stomach, prostate, bladder, lung and uterus.

Bibliographic details of the publications referred to by author in this specification are collected at the end of the description.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

The increasing incidence of colorectal cancer has resulted in an ever increasing burden of illness in society. Further, half of all patients diagnosed with bowel cancer die within five years of diagnosis. Despite limited advances in chemotherapy, immunotherapy and radiotherapy for the treatment of metastatic colorectal cancer during the last few decades, there has been little overall improvement in the survival rate of patients diagnosed with colorectal cancer.

Little is known of the factors which regulate the growth of colon cancer. In particular, little is known of the specific cellular molecules involved in the unregulated growth of neoplastic cells. For example, the expression on some neoplastic cells of tumour specific molecules (for example oncofoetal antigens) does not necessarily indicate the existence of a relationship between expression of these molecules and up-regulation of cell growth. In fact, the abnormal expression of cell surface molecules by neoplastic cells can be a secondary consequence of

the mechanism which has rendered the cell neoplastic. The identification of molecules involved in unregulated growth of neoplastic cells is further complicated by the fact that even where a correlation is found to exist between inducing the expression of a molecule on a cell and increasing the growth of that cell, down regulating expression of this molecule on a neoplastic cell will not necessarily lead to a reduction in the neoplastic cell's growth. Accordingly, there is a need to identify molecules directly involved in the regulation of neoplastic cell growth to determine their mechanism of action and to develop methods of modulating the functional activity of these molecules such that regulation of neoplastic cell growth can be effected.

In work leading up to the present invention, the inventors have surprisingly determined that the down-regulation of integrin expression, and in particular αvβ6 expression, on neoplastic cells leads to inhibition of the growth of these neoplastic cells. Further, the inventors have determined the mechanism by which αvβ6 acts to regulate cell growth. Specifically, whereas no MAP kinase or other subunit form of MAP kinase has been shown to bind any of the 23 integrins, the inventors have surprisingly determined that MAP kinase binds directly to the cytoplasmic β6 domain of αvβ6. The identification of this functional relationship permits the rational design of drugs for therapeutically or prophylactically modulating the functional activity of integrins expressed by neoplastic cells, such as αvβ6, and the identification of a range of molecules for use in the modulation of said functional activity.

Accordingly, one aspect of the present invention is directed to a method of modulating the growth of a cell, said method comprising contacting said cell with an agent for a time and under conditions sufficient to modulate the functional activity of an integrin molecule.

More particularly, the present invention is directed to a method of modulating the growth of a neoplastic cell, said method comprising contacting said cell with an agent for a time and under conditions sufficient to modulate the functional activity of an integrin molecule.

Reference to "modulating" should be understood as a reference to up-regulating or down-regulating the growth of a cell or the functional activity of an integrin molecule. More

specifically, reference to "down regulating" should be understood as a reference to preventing, reducing or otherwise inhibiting one or more aspects of the growth of a cell or of the functional activity of the subject integrin. Although the preferred method is to down-regulate the growth of neoplastic cells, for example as a therapeutic treatment for cancer, it may also be desirable to up-regulate cell growth. For example, it may be desirable to immortalise a population of cells, *in vitro*, to facilitate their long term *in vitro* use or, for example, to facilitate the *in vitro* growth of tissues such as skin.

Accordingly, the present invention still more particularly provides a method of down-regulating the growth of a neoplastic cell said method comprising contacting said cell with an agent for a time and under conditions sufficient to down-regulate the functional activity of an integrin molecule wherein down-regulation of the functional activity of said integrin molecule down- regulates the growth of said neoplastic cell.

Reference to an "integrin" molecule should be understood as a reference to any molecule the functional activity of which includes binding to matrix ligands and cytokines. Further, the integrin molecule of the present invention is one, the functional activity of which, either directly or indirectly exhibits a role in the growth of a cell. The integrins represent a distinct structural family of adhesion molecules. In a preferred embodiment, the integrin is $\alpha v\beta 6$ or its functional equivalent or derivative. To the extent that it is not specified, reference to an integrin, in general, or $\alpha v\beta 6$ should be understood to include reference to functional equivalents and derivatives thereof.

According to this preferred embodiment, the present invention is directed to a method of down- regulating the growth of a neoplastic cell, said method comprising contacting said cell with an agent for a time and under conditions sufficient to down-regulate the functional activity of $\alpha v\beta 6$ or its functional equivalent or derivative wherein down-regulation of the functional activity of said $\alpha v\beta 6$ down-regulates the growth of said neoplastic cell.

Reference to a "neoplastic cell" should be understood as a reference to a cell exhibiting abnormal "growth". The term "growth" should be understood in its broadest sense and

includes reference to proliferation. In this regard, an example of abnormal cell growth is the uncontrolled proliferation of a cell. The neoplastic cell may be a benign cell or a malignant cell. Preferably, the cell is malignant. To the extent that the present invention is directed to the down-regulation of αvβ6, the neoplastic cell is preferably a malignant epithelial cell. Without limiting the theory or mode of operation of the present invention in any way, αvβ6 is found to be predominantly expressed by neoplastic epithelial cells. Accordingly, modulation of the functional activity of this particular integrin molecule provides the advantage of down-regulating neoplastic cell growth in a cell specific manner. In one particular embodiment the neoplastic cell is a malignant epithelial cell which is derived from the colon, breast, skin, stomach, prostate, bladder, lung or uterus. Even more particularly, the malignant epithelial cell is a malignant colorectal cell.

It should be understood that the cell which is treated according to the method of the present invention may be located *ex vivo* or *in vivo*. By *"ex vivo"* is meant that the cell has been removed from the body of a subject wherein the modulation of its growth will be achieved *in vitro*. For example, the cell may be a non-neoplastic cell which will be immortalised by up-regulating αvβ6 expression. In accordance with the preferred aspects of the present invention, the cell may be a neoplastic cell, such as a malignant cell, located *in vivo* (such as in the colon) and the down-regulation of its growth will be achieved by applying the method of the present invention *in vivo*. It should also be understood that where reference is made to a specific cell type which is located *in vivo*, such as a malignant colorectal cell, this cell may be located in the colorectal area of the patient or, if a colorectal primary malignancy has metastasised, the subject colorectal cell may be located in another region of the patient's body. For example, it may form part of a secondary tumour (metastasis) which is located, for example, in the liver, lymph node or bone.

According to this preferred embodiment, the present invention provides a method of down-regulating the growth of a malignant epithelial cell, said method comprising contacting said cell with an agent for a time and under conditions sufficient to down-regulate the functional activity of αvβ6 or its functional equivalent or derivative wherein down-regulating the functional activity of said αvβ6 down-regulates the growth of said epithelial cell.

Even more preferably, the present invention provides a method of down-regulating the growth of a malignant colorectal epithelial cell, said method comprising contacting said cell with an agent for a time and under conditions sufficient to down-regulate the functional activity of αvβ6 or its functional equivalent or derivative wherein down-regulating the functional activity of said αvβ6 down-regulates the growth of said colorectal epithelial cell.

Without limiting the invention to any one theory or mode of action, the integrins are trans-membrane molecules. Accordingly, they form a bridge between extracellular matrix located outside the cell and the various proteins located inside the cell. These intracellular proteins include enzymes termed kinases, some of which enter the nucleus and switch on growth-promoting genes. αvβ6 has been determined to stimulate epithelial cell growth by binding a kinase responsible for transmitting growth signals to the nucleus. Specifically, αvβ6, the expression of which is up-regulated on cancerous cells but not normal cells, becomes highly expressed in malignancies. It is thought that the cytoplasmic domain of the β6 integrin subunit binds a member of the mitogen activated protein (MAP) kinase family of enzymes termed extracellular signal-related kinase 2 ($ERK_2$). This is a surprising finding since the MAP kinase has never been shown to bind an integrin molecule. Further, down-regulation of αvβ6 expression is associated with a decrease in MAP kinase activity, which activity is dependent on the presence of a cytoplasmic domain of the β6 integrin subunit. In a particularly surprising development, the inventors have determined that whereas insertion of the αvβ6 gene can increase cell growth, the down-regulation of expression of this molecule decreases cell growth more significantly than the extent to which insertion of the gene increases cell growth. This supports the observations that a direct correlation between the up and down-regulation of expression of a molecule and its effect on cell growth cannot be assumed. It is thought that targeting αvβ6 mediated MAP kinase activity by inhibiting the β6-$ERK_2$ interaction will avoid unwanted side effects in non-malignant cells which lack αvβ6 expression, since MAP kinase activity is required for maintenance of cellular functions within normal cells. Down-regulation of the functional activity of αvβ6 can therefore be achieved by:

(i) preventing or down-regulating the expression of the αvβ6 molecule; or

(ii) inhibiting the signalling mechanism of αvβ6.

To the extent that up-regulation of cell growth may be required, this may be achieved, for example, by up-regulating either the expression of αvβ6 or its signalling capabilities.

Modulation of the functional activity of αvβ6 (or other integrin molecule) can be achieved by any one of several techniques, including but in no way limited to, introducing into said cell a proteinaceous or non-proteinaceous molecule which:

(i) modulates the expression of αvβ6 DNA (for example, by modulating transcription of the genomic αvβ6 DNA or transcription of the αvβ6 mRNA)

(ii) functions as an antagonist of αvβ6

(iii) functions as an agonist of αvβ6.

The proteinaceous molecule may be derived from natural or recombinant sources including fusion proteins, peptides or following, for example, natural product screening. For example, ELISA assays may be established which provide a method of large scale screening of molecules which bind to the β6-$ERK_2$ interaction site. For example, a molecule which competes with the cytoplasmic domain of β6 for binding to $ERK_2$. This method may be utilised to screen for both agonists and antagonists of β6 mediated signalling. Said non-proteinaceous molecule may be, for example, a nucleic acid molecule or may be derived from natural sources, such as for example, natural product screening or may be chemically synthesized. The present invention contemplates chemical analogs of αvβ6 or small molecules capable of acting as agonists or antagonists of αvβ6. Chemical agonists may not necessarily be derived from αvβ6 but may share certain formational similarities. Alternatively, chemical agonists may be specifically designed to mimic certain physio-chemical properties of αvβ6. Antagonists may be any compound capable of blocking, inhibiting or otherwise preventing αvβ6 from carrying out its normal biological function. For example, antagonists of the β6-$ERK_2$ interaction may be designed based on the tertiary

structure of the β6 cytoplasmic domain and/or $ERK_2$ as determined by nuclear magnetic resonance and/or x-ray crystallography. Antagonists include monoclonal antibodies and antisense nucleic acids which prevent transcription or translation of αvβ6 genes or mRNA in mammalian cells. Modulation of αvβ6 expression may also be achieved utilising antigens, RNA, ribosomes, DNAzymes, RNA aptamers or antibodies.

The proteinaceous or non-proteinaceous molecule may act either directly or indirectly to modulate the expression of αvβ6 DNA or mRNA or the activity of αvβ6 protein. Said molecule acts directly if it associates with αvβ6 to modulate its expression or activity. Said molecule acts indirectly if it associates with a molecule other than αvβ6 nucleic acid molecule or αvβ6 expression product which other molecule either directly or indirectly modulates the expression or activity of αvβ6. Accordingly, the method of the present invention encompasses the regulation of αvβ6 nucleic acid expression or αvβ6 expression product functional activity via the induction of a cascade of regulatory steps.

In one embodiment of the present invention, the object of the present invention - being inhibition of the functional activity of αvβ6 - is achieved by inhibiting the functional activity of the β6 subunit.

Down-regulation of the functional activity of the β6 subunit may be achieved, for example, utilising antisense nucleic acid sequences which are introduced into a neoplastic cell to prevent expression of the β6 subunit. For example, expression of a nucleic acid construct comprising β6 DNA inserted in the reverse orientation results in the synthesis of β6 antisense RNA which, via its hybridisation to β6 mRNA, prevents translation of the β6 mRNA. The nucleic acid construct utilised in accordance with this embodiment of the present invention may be any suitable construct but is preferably a viral construct into which is inserted, in reverse direction, a nucleic acid sequence corresponding to all or part of the coding region of the β6 gene (Sheppard, D. *et al.*, (1990)). Viral vectors suitable for use in the present invention include, but are not limited to, adenovirus, vaccinia virus, retroviruses, BCG, *E.Coli*, pox viruses, herpes virus or polio virus. Preferably, the viral vector is a non-replicating adenoviral shuttle vector comprising the polyadenylation site and elongation factor 1-α

promoter. In another preferred embodiment the viral vector is a replication-deficient adenovirus which has been generated via the use of cytomegalovirus.

Accordingly, in a preferred embodiment there is provided a method of down-regulating the growth of a neoplastic epithelial cell, said method comprising contacting said cell with an effective amount of a first nucleic acid molecule or functional derivative, chemical equivalent, analogue or homologue thereof which is capable of interacting with a second nucleic acid molecule, or which first nucleic acid molecule is capable of translation to a nucleic acid molecule capable of interacting with a second nucleic acid molecule, which second nucleic acid molecule is translatable to all or part of an amino acid sequence of β6 whereby said interaction facilitates a reduction in the functional activity of said β6.

Reference to said first "nucleic acid molecule" should be understood as a reference to any molecule comprising a nucleotide sequence which molecule either directly or indirectly facilitates reduction, inhibition or other form of down-regulation of the functional activity of αvβ6, and more particularly β6. Examples of nucleic acid molecules which fall within the scope of this definition include, but are not limited to:

- oligonucleotides, such as antisense oligonucleotides, which interact with a nucleic acid sequence encoding αv or β6, a transcription or translation regulatory factor or a promoter thereby inhibiting expression of αv or β6, the transcription or translation regulatory factor or the functioning of the promoter.

- nucleic acid molecules which encode all or part of a αv or β6 or a transcription or translation regulatory factor wherein co-suppression of the expression of αv or β6 or transcription or translation regulatory factor is induced.

- ribozymes, the hybridisation component of which interacts with a nucleic acid molecule encoding a αv or β6, or transcription or translation regulatory factor

or a promoter and the catalytic component of which cleaves this nucleic acid molecule.

Preferably said first nucleic acid molecule is an oligonucleotide and said second nucleic acid is mRNA. Even more preferably, said first nucleic acid molecule comprises a viral shuttle vector into which has been inserted β6 DNA inserted in the reverse orientation.

Reference to "functional derivatives, chemical equivalents, analogues or homologues" of a nucleic acid molecule should be understood to include reference to fragments, parts, portions, chemical equivalents, mutants and mimetics from natural, synthetic or recombinant sources exhibiting any one or more of the functional activities of said nucleic acid molecule.

Derivatives of nucleic acid sequences may be derived from single or multiple nucleotide substitutions, deletions and/or additions including fusion with other nucleic acid molecules or non-nucleic acid molecules. The derivatives of said nucleic acid molecules include fragments having particular epitopes or parts of the nucleotide or nucleic acid sequence fused to other proteinaceous or non-proteinaceous molecules. Analogues contemplated herein include, but are not limited to, modifications to the nucleotide or nucleic acid molecules such as modifications to its chemical makeup or overall conformation. This includes, for example, modification to the manner in which the nucleotide or nucleic acid molecule interacts with other nucleotides or nucleic acid sequences, for example, at the level of backbone formation or complementary base pair hybridisation.

Reference to "functional equivalent or derivative" of an integrin should be understood to include reference to fragments, parts, portions, equivalents and mimetics which exhibit one or more of the functional activities of the integrin. Derivatives may be derived from insertion, deletion or substitution of amino acids. Insertional amino acid sequence variants are those in which one or more amino acid residues are introduced into a predetermined site in the protein, for example, random insertion. Deletional variants are characterised by the removal of one or more amino acids from the sequence. Substitutional amino acid variants are those in which at least one residue in the sequence has been removed and a different

residue inserted in its place. Additions to amino acid sequences include fusions with other peptides, polypeptides and proteins. Amino acid insertional derivatives include amino and/or carboxylic terminal fusions as well as intrasequence insertion of single or multiple amino acids. In addition to integrin derivatives which may be naturally expressed *in vivo*, the method of the present invention also contemplates recombinantly produced integrin derivatives, the encoding nucleic acid sequence of which may have been introduced into the body for the treatment or prophylaxis of an unrelated disease condition or for some other purpose.

Reference to "interacting" should be understood as a reference to any form of interaction such as hybridisation between complementary nucleic acid base pairs or some other form of interaction such as the formation of bonds between any portion of the oligonucleotide with any portion of the RNA molecule. Said interaction may occur *via* the formation of bonds such as covalent bonds, hydrogen bonds, van der Waals forces or via any other mechanism of interaction.

In another embodiment of the present invention, down-regulation of the functional activity of $\alpha v\beta 6$ is achieved by inhibiting the $\alpha v\beta 6$-MAP kinase interaction utilising an agent, such as a peptide, which competes with the cytoplasmic region of the $\beta 6$ subunit for binding to $ERK_2$. Preferably, the agent competes with the sequence in $ERK_2$ responsible for binding to the $\beta 6$ cytoplasmic domain. Without limiting the invention to any one theory, it is thought that by preventing binding of $ERK_2$, signalling by $\alpha v\beta 6$ (which signalling is thought to include the signals required to stimulate growth) is inhibited. Effecting internalisation of these blocking molecules may require the coupling of said molecule to a "carrier peptide", such as penetratin. In addition to transporting the blocking molecule, per se, across the membrane of the neoplastic cell, the method of the present invention should be understood to extend to the use of genetic techniques such as the administration of vector molecules which encode a proteinaceous blocking molecule.

A further aspect of the present invention relates to the use of the invention in relation to mammalian disease conditions. For example, the present invention is particularly useful, but

in no way limited to, use in the treatment of primary and secondary tumours arising from epithelial malignancies such as those of the colon, breast, skin, stomach, prostate, bladder, lung and uterus.

Accordingly, the present invention contemplates a method for the treatment and/or prophylaxis of a neoplastic conditions in a mammal said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to down-regulate the functional activity of an integrin molecule wherein the down-regulation of the functional activity of said integrin molecule down-regulates the growth of the neoplastic cells.

More particularly, the present invention provides a method for the treatment and/or prophylaxis of an epithelial malignancy in a mammal said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to down-regulate the functional activity of $\alpha v\beta 6$ or functional equivalent or derivative wherein the down-regulation of the functional activity of said $\alpha v\beta 6$ down-regulates the growth of the malignant epithelial cells.

In a most preferred embodiment, the epithelial malignancies comprise cells arising from the colon, breast, skin, stomach, prostate, bladder, lung and uterus.

Still more particularly, said agent is a $\beta 6$ antisense molecule or an inhibitor of the $\beta 6$-$ERK_2$ binding interaction.

Reference herein to "treatment" and "prophylaxis" is to be considered in its broadest context. The term "treatment" does not necessarily imply that a mammal is treated until total recovery. Similarly, "prophylaxis" does not necessarily mean that the subject will not eventually contract a disease condition. Accordingly, treatment and prophylaxis include amelioration of the symptoms of a particular condition or preventing or otherwise reducing the risk of developing a particular condition. The term "prophylaxis" may be considered as reducing the severity of onset of a particular condition. "Treatment" may also reduce the severity of an

existing condition or the frequency of acute attacks (for example, reducing the frequency of acute asthma attacks).

The subject of the treatment or prophylaxis is generally a mammal such as but not limited to human, primate, livestock animal (e.g. sheep, cow, horse, donkey, pig) companion animal (e.g. dog, cat) laboratory test animal (e.g. mouse, rabbit, rat, guinea pig, hamster) captive wild animal (e.g. fox, deer). Preferably the mammal is a human or primate. Most preferably the mammal is a human.

In accordance with these methods, the agents herein defined may be coadministered with one or more other compounds or molecules. For example, antisense β6 vectors may be administered in combination with chemotherapeutic agents. Alternatively, the proteinaceous or non-proteinaceous molecules utilised to inhibit the β6-$ERK_2$ subunit binding interaction may be administered in conjunction with antisense β6 therapy and/or chemotherapeutic agents. By "coadministered" is meant simultaneous administration in the same formulation or in two different formulations via the same or different routes or sequential administration by the same or different routes. By "sequential" administration is meant a time difference of from seconds, minutes, hours or days between the administration of the two types of molecules. These molecules may be administered in any order.

A further aspect of the present invention relates to the use of an agent in the manufacture of a medicament for the treatment of a neoplastic condition wherein said agent down-regulates the functional activity of an integrin.

Preferably said integrin is αvβ6.

In yet another further aspect, the present invention contemplates a pharmaceutical composition comprising an agent capable of modulating the functional activity of αvβ6 or functional equivalent or derivative thereof, as hereinbefore defined, together with one or more pharmaceutically acceptable carriers and/or diluents. Said agents are referred to as the "active ingredients".

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions (where water soluble) and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as licithin, by the maintenance of the required particle size in the case of dispersion and by the use of superfactants. The preventions of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimersal and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredient into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze-drying technique which yield a powder of the active ingredient plus any additional desired ingredient from previously sterile-filtered solution thereof.

When the active ingredients are suitably protected they may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, or it may be enclosed in hard or soft shell gelatin capsule, or it may be compressed into tablets, or it may be incorporated directly with the food of the diet. For oral therapeutic administration, the active

compound may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 1% by weight of active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 5 to about 80% of the weight of the unit. The amount of active compound in such therapeutically useful compositions in such that a suitable dosage will be obtained. Preferred compositions or preparations according to the present invention are prepared so that an oral dosage unit form contains between about 0.1 $\mu$g and 2000 mg of active compound.

The tablets, troches, pills, capsules and the like may also contain the following: A binder such as gum tragacanth, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such a sucrose, lactose or saccharin may be added or a flavouring agent such as peppermint, oil of wintergreen, or cherry flavouring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain the active compound, sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavouring such as cherry or orange flavour. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compound may be incorporated into sustained-release preparations and formulations.

Pharmaceutically acceptable carriers and/or diluents include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, use thereof in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the novel dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active material and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active material for the treatment of disease in living subjects having a diseased condition in which bodily health is impaired.

The principal active ingredient is compounded for convenient and effective administration in effective amounts with a suitable pharmaceutically acceptable carrier in dosage unit form as hereinbefore disclosed. A unit dosage form can, for example, contain the principal active compound in amounts ranging from 0.5 $\mu$g to about 2000 mg. Expressed in proportions, the active compound is generally present in from about 0.5 $\mu$g to about 2000 mg/ml of carrier. In the case of compositions containing supplementary active ingredients, the dosages are determined by reference to the usual dose and manner of administration of the said ingredients.

The pharmaceutical composition may also comprise genetic molecules such as a vector capable of transfecting target cells where the vector carries a nucleic acid molecule capable of modulating integrin expression or integrin activity. The vector may, for example, be a viral vector as hereinbefore defined.

Administration of the agent in the form of a pharmaceutical composition may be performed by any convenient means. The agent of the pharmaceutical composition is contemplated to exhibit therapeutic activity when administered in an amount which depends on the particular case. Variation depends for example, on the human or animal and the agent chosen. Dosage regimes may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily, weekly, monthly or other suitable time intervals or

the dose may be proportionally reduced as indicated by the exigencies of the situation. The agent may be administered in any suitable manner. Routes of administration include, but are not limited to, respiratorally, intratracheally, nasopharyngeally, intravenously, intraperitoneally, subcutaneously, intracranially, intradermally, intramuscularly, intraoccularly, intrathecally, intracereberally, intranasally, infusion, orally, rectally, *via* IV drip patch and implant. With respect to intravenous routes, particularly suitable routes are via injection into vessels which supply the tumour or diseased organs. Peptides may also be installed into cavities for example the pleural or peritoneal cavity or injected directly into tumour tissues.

Further features of the present invention are more fully described in the following non-limiting Figures and/or Examples.

## BRIEF DESCRIPTION OF THE DRAWINGS

**Figure 1** is a graphical representation of:

(A) Surface biotinylation and immunoprecipitation of integrin subunits β5 and β6 in HT29 colon cancer cells stably transfected with either vector alone (mock transfectants) or antisense β6 construct. Down-regulation of αvβ6 expression in antisense β6 transfectants is accompanied by increased expression of αvβ6.

(B) Effect of antisense β6 in HT29 cells on tumour growth *in vivo*. Tumour cells from HT29 mock and antisense β6 transfectants were injected subcutaneously into the flanks of athymic mice and tumour diameters measured weekly. A progressive increase in tumour growth was observed for mock transfectants in contrast to cell deficient in αvβ6.

(C) Immunoprecipitation of cell lysates from the colon cancer cell lines SW480 β6 and WiDr with anti-β6 antibody followed by Western blotting with an antibody that recognises phosphorylated $ERK_2$. The position of recombinant $ERK_2$ is co-immunoprecipitated with β6 but not with the β5 integrin subunit or in control immunoprecipitations (against human immunoglobulin). Moreover, in the human colon cancer cell line, WiDr, which has been stably transfected with antisense β6 construct to down-regulate constitutive β6 expression, markedly less $ERK_2$ co-immunoprecipitates with β6 than in stably transfected WiDr cells containing vector only (mock transfectants which express normal levels of β6). Similarly, heterologous expression of β6 in the SW480 cell line which lacks constitutive β6 expression, results in β6-$ERK_2$ association.

(D) Effect of antisense β6 transfection in HT29 and WiDr cell lines on the ability of cell lysates to phosphorylate myelin basic protein in a standard MAP kinase activity assay. For the cell lines HT29 and WiDr, down-regulation of constitutive β6 expression

results in diminished MAP kinase activity compared with activity in lysates from mock transfectants expressing vector alone.

(E) Heterologous expression of αvβ6 in the SW480 colon cancer cell line is associated with enhanced MAP kinase activity in contrast to cells transfected with vector alone (mock) or with truncated β6 lacking the entire cytoplasmic domain.

(F) *In vitro* proliferation of HT29 wild-type cells after four days in culture in the continuous presence of the MAP kinase (MEK) inhibitor PD98059 or DMSO (control). Proliferation was assessed by $^{3}$H-thymidine uptake.

**Figure 2** is a photographic representation of athymic mice six weeks following subcutaneous injection of $10^{6}$ cells of HT29 mock transfectant (A) or antisense β6 transfectant (B).

## EXAMPLE 1

## WiDr and HT29 GENE THERAPY ANALYSES

WiDr and HT29, human colon cancer cell lines, constitutively express αvβ6. Transfection of these cells with a non-adenoviral vector containing β6 in the anti-sense orientation results in marked downregulation of αvβ6 expression (Figure 1A). Downregulation of β6 in these stably-transfected cell lines lead to a dramatic reduction in cell proliferation *in vitro* and tumour growth *in vivo* as shown for HT29 cells in Figure 1B and Figure 2 (left mock transfectant and right anti-sense β6).

## EXAMPLE 2

## GENERATION OF RECOMBINANT REPLICATION-DEFICIENT ADENOVIRUS

Recombinant, replication-deficient adenovirus containing β6 in the anti-sense and sense orientation are generated using the AdEasy vector system (He *et al.* 1998) by inserting the gene construct as a cassette, comprising the polyadenylation site and elongation factor 1-alpha promoter, into a shuttle vector. The choice of a different promoter driving anti-sense β6 to that available in the shuttle vector is based on success in down-regulating αvβ6 expression in colon cancer cell lines resulting in inhibition of tumour growth. Appropriate insertion of the cassette containing either anti-sense β6 or sense β6 is confirmed by restriction endonuclease digest. Function of the shuttle vectors is confirmed by means of transient transfections of 293 cells and colon cancer cell lines and assessment of αvβ6 expression (FACScan analyses, β6 immunoprecipitations and Northern analysis). To confirm that the adenovirus preparations are comprised of only replication-deficient adenoviruses, HeLa cells are infected with recombinant virus at a multiplicity of infection of 100pfu/cell and viral DNA analysed by polymerase chain reaction (PCR) following repeated rounds of HeLa cell infection in which supernatant prepared from cell extracts of the first round of infected HeLa cells is used to infect the next batch of HeLa cells on two successive occasions (Zhang *et al.*, 1995). If the viruses are able to replicate through infection, then viral amplification is detectable in the second or third round of infection by examination of cytopathic effects and PCR analysis of viral DNA. To further exclude the possibility of viral DNA replication,

human non-small cell lung carcinoma H358 cells are infected with recombinant β6 gene-containing virus and cell cultures labelled with $^{32}P$ followed by extraction of low-molecular weight DNA, endonuclease digestion, and analysis of $^{32}P$-labelled DNA in an agarose gel. Gels are dried and exposed to x-ray film and wild-type adenovirus used as a positive control.

## EXAMPLE 3
## *IN VITRO* GENE DELIVERY

*In vitro* gene transduction of colon cancer cell lines WiDr and HT29, which constitutively express αvβ6, is performed by PLATING cells in 6-well culture plates followed by incubation with recombinant virus expressing anti-sense β6 48 hours after plating. As controls, cells are exposed to phosphate buffered saline (PBS) alone and recombinant virus expressing β6 in the sense orientation. Gene expression within the transduced cell lines is assessed by means of FACScan analyses, β6 immunoprecipitations and Northern analysis. *In vitro* cell proliferation is measured by cell counts and $^{3}H$-thymidine incorporation for cells cultured as monolayers and within 3-dimensional collagen matrices. The effect of gene transduction on apoptosis is evaluated by means of propidium iodide staining as described by Thomas and Hersey (1998).

## EXAMPLE 4
## *IN VIVO* GENE DELIVERY

(a) Toxicity Studies: Neither intra-cardiac injection, intra-tracheal nor intra-tumour inoculation of recombinant, replication-deficient adenovirus has been associated with significant toxicity (Tang *et al.*, 1994; Zhang *et al.*, 1995; Spitz *et al.*, 1996). Nevertheless, adenoviral toxicity *in vivo* is assessed. Twenty-five athymic mice are divided into 5 groups of 4 mice per group; each group receives one of four escalating dosages, $10^7$, $10^8$, $10^9$ and $10^{10}$ pfu/mouse or PBS (control). Two mice from each group receive injections of replication-deficient anti-sense adenovirus β6 into the tail vein and two from each group receive adenovirus by subcutaneous route injected into the flank. The liver has been reported to be the major organ of gene transfer and expression after systemic administration of recombinant adenoviruses to animals (Li

*et al.*, 1993). Systemic effects (skin colour, respiration, mouse activity and behaviour) are observed and recorded daily after injection. One mouse from each dose/injection protocol is autopsied on day 3 and the other on day 21 after injection and tissues harvested for histological analysis. Formalin-fixed, paraffin-embedded sections from the liver, heart, brain, lung, kidney and subcutaneous tissue in the region of the inoculation site are examined and graded for signs of inflammation (not seen, mild, moderate or severe).

(b) Subcutaneous tumorigenicity assay: WiDr and HT29 colon cancer cells are injected subcutaneously into the flanks of athymic mice and 10 mice studied in each group. Adenovirus is inoculated into the developing tumour xenografts between day 10 and 14 after tumour injection, namely, when tumours have reached approximately 5mms. in diameter. Tumour growth is followed by measuring tumour diameters with calipers and excised tumours are also weighed at the termination of experiments at 6 weeks. The number of mice chosen is based on a 50% reduction in the tumour size for mice treated with adenovirus anti-sense β6 compared with the sense β6 vector. Given that tumours at 6 weeks reach, on average, 15 mm in diameter, 10 mice are required for each group to detect a 50% reduction in tumour size at a power of 90% and a significance level of $\alpha=0.05$ assuming a standard deviation for tumour size of 33%. Adenovirus is administered in three divided doses given every other day for a total viral dose of $10^{10}$ pfu (in the absence of toxic effects), and each divided dose suspended in a volume of 100$\mu$l PBS is injected directly into the tumours by means of a single pass of a 27-gauge hypodermic needle.

(c) Liver metastasis model: HT29 colon cancer cells are injected into the spleen of athymic mice via mini-laparotomy since intra-splenic tumour cell inoculation results in the rapid clearance (within a few hours) of the cells to the liver via the portal venous circulation. Adenovirus is injected ($10^{10}$ pfu/mouse if confirmed to be non-toxic in preliminary studies, otherwise an appropriately lower dose) into the tail vein 10-14 days after intra-splenic tumour cell injection and tumour burden in the liver evaluated at 18-20 weeks. Mice are injected by PBS only, adenovirus sense β6 and

adenovirus anti-sense β6 and 45 mice are used in each group. The choice of 45 mice is based on a 35% incidence of liver metastases following intrasplenic inoculation of HT29 cells (J. Reeder, Master of Science Thesis, University of Queensland, 1997). The proportion of mice completely tumour free as well as the number of visible tumours per mouse will be recorded and 45 mice per group is an appropriate number to detect a 70% reduction in the number of mice completely free of hepatic metastases following anti-sense β6 gene therapy at a power of 80% and significance level of $\alpha=0.05$.

## EXAMPLE 5

## IDENTIFICATION OF THE β6 CYTOPLASMIC DOMAIN BINDING SITE FOR $ERK_2$

The β6 subunit cytoplasmic domain binds a member of the mitogen-activated protein kinase (MAPk) family of enzymes called extracellular signal-related kinase-2 ($ERK_2$). The subfamily of ERKs are serine/threonine kinases which are activated by phosphorlation of tyrosine and threonine residues by upstream enzymes called MAP kinase kinases (MEKs) (Payne *et al*, 1991). The technique of phage display using of $\gamma GT^{11}$ cDNA library derived from a colon cancer cell line has been used to identify proteins which interact with the cytoplasmic domain of the β6 integrin subunit. The ERK-like interacting fragment identified displayed 100% amino acid identity with $ERK_2$ and Western blotting for $ERK_2$ following β6 immuno-precipitation from colon cancer cells confirmed the β6-$ERK_2$ interaction (Figure 1C). Moreover, this binding is specific for β6 and does not involve the β5 integrin subunit as shown in Figure 1C. Downregulation of αvβ6 expression in the WiDr cell line (by means of anti-sense β6) results in markedly less $ERK_2$ co-immunoprecipitated with β6 than for cels stably transfected with vector alone (Figure 1C) together with a reduction in MAP kinase activity as assessed in a standard MAP kinase activity assay (Figure 1D). Moreover, heterologous expression of the full length β6 subunit in SW480 colon cancer cells which lack constitutive αvβ6 expression increases MAP kinase activity whereas no increase in activity is observed for stable transfectants expressing the truncated form of β6 lacking the cytoplasmic domain (Figure 1E). By specifically targetting the β6-$ERK_2$ interaction instead

of targetting total MAP kinase activity within a cell, with, for example, the MEK inhibitor PD98059, which effectively inhibits cell proliferation *in vitro* as shown in Figure 1F, unwanted side effects in non-malignant cells (which lack αvβ6 expression) are avoided due to the requirement for MAL kinase activity in maintaining many cellular functions within normal cells.

The binding site(s) for $ERK_2$ are determined by screening overlapping peptides in an enzyme-linked immunosorbent assay (ELISA) format. Synthetic peptides 20 amino acids long which overlap by 10 amino acids are prepared based on the 52 amino acid long β6 cytoplasmic domain and the 20-mer peptides biotinylated at the amino-terminus (Auspep, Melbourne, Australia). In brief, purified, recombinant $ERK_2$ (New England Biolabs) or bovine serum albumin is coated on to 96-well culture plates for 2 hours at 37°C, after which time remaining sites are blocked with 1% gelatin as described by Greenway and colleagues (1996). After washing, wells are incubated for 2 hours at 37°C with the biotinylated peptides, washed thoroughly and incubated with streptavidin-HRP and substrate (o-phenylenediamine; Sigma) prior to measuring absorbance in a plate reader.

In another approach the overlapping biotinylated peptides (5μmol diluted in PBS) are coated onto streptavidin-coated 96-well plates and following washing with PBS-Tween, the remaining binding sites blocked with 1% gelatin. Peptide-coated wells are be incubated with purified, recombinant $ERK_2$, washed to remove unbound $ERK_2$ and treated with mouse anti-$ERK_2$ or an isotype control antibody. Binding of $ERK_2$ to biotinylated peptide is detected by exposing wells to HRP-conjugated goat anti-mouse antibody followed by substrate and measurement of absorbance in a plate reader. Control for the binding assay includes incubation and the immobilised peptides with PBS-Tween alone. To confirm peptide-$ERK_2$ binding, $ERK_2$ are pre-incubated with the relevant peptide for one hour at 4°C before adding to immobilised peptide in the wells. The overlapping peptides are tested over a range of concentrations and depending on the results obtained, a further series of 10-mer overlapping peptides are tested over a range of concentrations. Putative blocking peptides are also tested in non-biotinylated form for their ability to inhibit binding of $ERK_2$ to the immobilised biotinylated 52-mer peptide (the full-length β6 cytoplasmic domain) in a concentration-dependent manner.

Scrambled peptides serve as controls. Affinity precipitation is also performed to confirm the binding interaction between β6 and $ERK_2$. Biotinylated peptides are precoupled to agarose-streptavidin beads and the beads incubated with cell lysates from β6-expressing and non-expressing SW480 colon cancer cells. Samples are analysed by means of SDS-PAGE, nitrocellulose transfer and immunoblotting using anti-$ERK_2$ antibody.

## EXAMPLE 6
## INTRACELLULAR DELIVERY OF PEPTIDE

Most peptides are poorly taken up by cells since they do not efficiently cross the lipid bilayer of the plasma membrane. In the past several years, several peptides have been discovered which have the ability to deliver macro-molecules across cell membranes in an energy-independent manner (Prociantz, 1996). This opens up the possibility of both testing new bioactive compounds in cell culture without drastically altering the cell membrane integrity, and of potentially delivering macro-molecular bioactive compounds *in vivo*. The peptides that have already been reported as being "carrier peptides" include penetration and variations thereof (Derossi *et al.* 1994, 1996), a human immunodeficiency virus Tat-derived peptide (Prociantz, 1996) and a peptide derived from a peptide called transportan (Pooga *et al.* 1998). Carrier peptides have been successfully used to facilitate internalisation of mimetics of Src homology 2 binding sites, and peptides which inhibit protein kinase C-mediated axon development and CD44 (hyaluronate receptor)-dependent migration (Théodore *et al.* 1995; Williams *et al.* 1997; Peck and Isacke, 1998; Derossi *et al.*, 1998).

Of importance to the development of a pharmaceutical tool such as a "carrier peptide" to deliver cargo peptides into cell is lack of toxicity of the "carrier peptide". Hence, prior to preparation of fusion peptide ("carrier" plus β6-$ERK_2$ inhibiting peptide), the toxicity profile for the various "carrier peptides" must be tested using normal and malignant cells. Potential toxicity of the "carrier peptides" is assessed by means of cell morphology, trypan-blue exclusion, assessment of apoptosis, and cell proliferation studies (cell counts, $^3$H-thymidine uptake and MTT assay).

## EXAMPLE 7

## *IN VITRO* FUNCTIONAL STUDIES

Cellular uptake of the fusion peptide is determined by means of coupling the blocking peptide to fluorescein for visualisation by confocal microscopy. The effect of β6-$ERK_2$ blocking peptide on binding between the β6 cytoplasmic domain and $ERK_2$ in β6-expressing cells (SW480β6, HT29, WiDr) are tested by means of β6 immunoprecipitations followed by Western blotting for any effect of blocking peptide on αvβ6 expression itself is monitored. Given that β6 expression is associated with enhanced MAP kinase activity (Figures 1D and E) the effect of the blocking peptide on the ability of β6-expressing cell lysates to phosphorylate myelin basic protein in a standard MAP kinase assay is examined. To exclude the possibility that the blocking peptide could bind to $ERK_2$ and mimic the action of β6, the effect of intracellular uptake of peptide on MAP kinase activity is assessed in SW480 wild-type cells which lack constitutive αvβ6 expression. In the unlikely event that a peptide(s) does act as a β6 mimic, an alternative strategy is adapted to identify binding regions on $ERK_2$ which interact with β6 by screening PCR-generated fragments of $ERK_2$ (corresponding to 100 nucleotides) using the Cyto Trap ™ two-hybrid system (Stratagene). The proliferative capacity of SW480β6 and mock (vector alone) transfectants and HT29/WiDr cell lines when exposed to blocking peptide over a dose range (using scrambled peptide as a control) are assessed for cells cultured as monolayers and within 3-dimensional collagen matrices by means of $^3H$-thymidine uptake and cell counts.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

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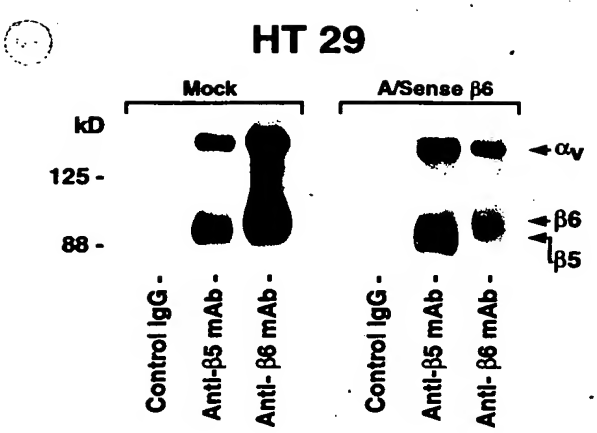
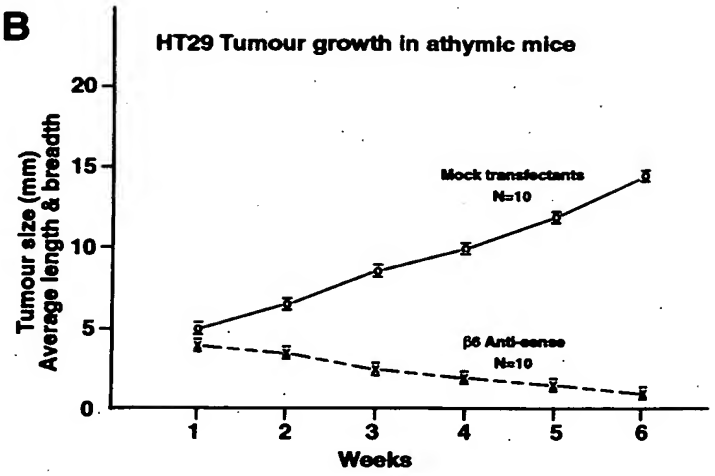
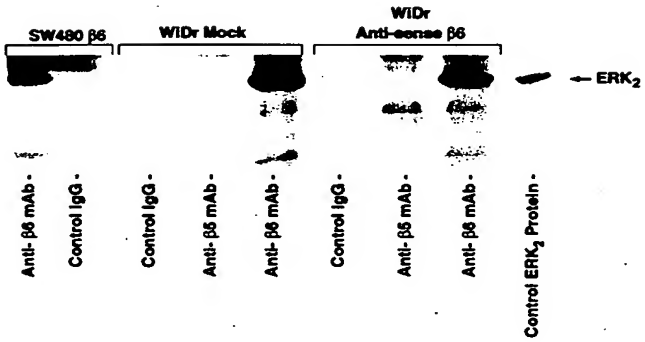
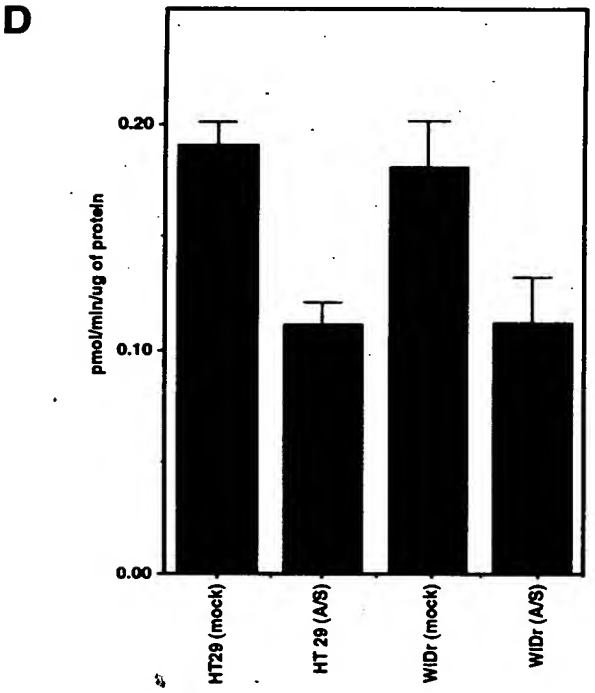
Zhang, W-W., Alemany, R. Wang, J., Koch, P.E., Ordonez, N.G. and Roth, J.A. *Human Gene Therapy 6:*155-164 (1995).

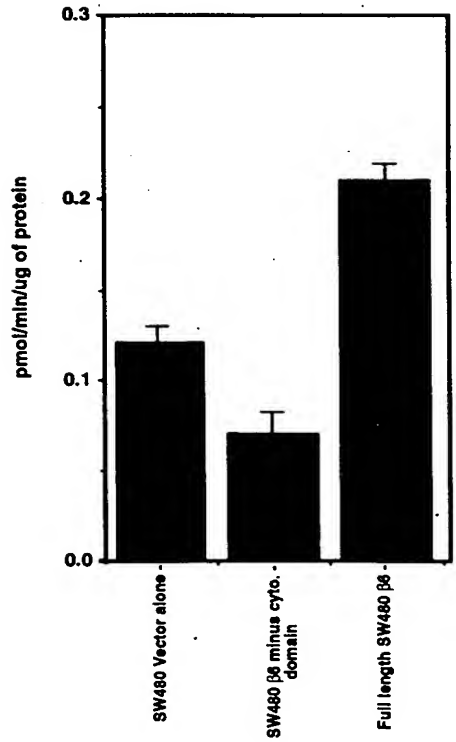
# FIGURE 1


HT 29
Mock
A/Sense β6
kD
125 -
88 -
αv
β6
β5
Control IgG -
Anti-β5 mAb -
Anti- β6 mAb -
Control IgG -
Anti-β5 mAb -
Anti- β6 mAb -


B
HT29 Tumour growth in athymic mice
Tumour size (mm)
Average length & breadth
Mock transfectants
N=10
β6 Anti-sense
N=10
Weeks


SW480 β6
WIDr Mock
WIDr
Anti-sense β6
ERK2
Anti- β6 mAb -
Control IgG -
Control IgG -
Anti- β5 mAb -
Anti- β6 mAb -
Control IgG -
Anti- β5 mAb -
Anti- β6 mAb -
Control ERK2 Protein -


D
pmol/min/ug of protein
HT29 (mock)
HT 29 (A/S)
WIDr (mock)
WIDr (A/S)


pmol/min/ug of protein
SW480 Vector alone -
SW480 β6 minus cyto. - domain
Full length SW480 β6 -

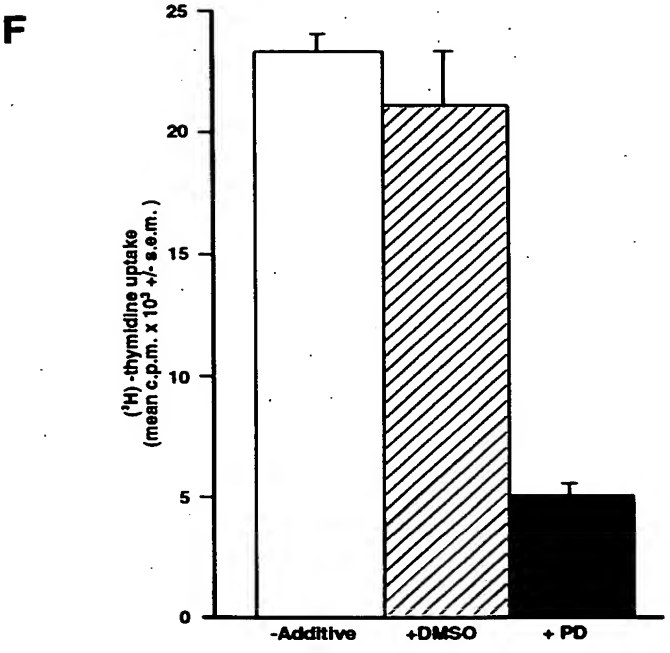

F
(3H) -thymidine uptake
(mean c.p.m. x 10³ +/- s.e.m.)
-Additive
+DMSO
+ PD

**FIGURE 2**